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The Effects of Acute Stress on Adrenal Ascorbic Acid Levels

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The Effects of Acute Stress on Adrenal Ascorbic Acid Levels

(TITLE)

BY

Robert Joseph Collier

THESIS

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A B S T R A C T

The diurnal rhythm of adrenal ascorbic acid (DAR) displays an inverse relationship to the diurnal rhythm of circulating levels of plasma corticosteroids. Alloxan monohydrate, 175 mg/kg body weight was given subcutaneously to albino rats to determine the effect of the diabetic state on the DAR. A total of 200 female albino rats were divided into two study groups. Study Group I was composed of 150 animals divided into populations of 15, each including 3 controls. Study Group II was composed of populations of 5, each group containing all controls or all alloxinated rats. The populations of 15 were sacrificed at 4-hour intervals for 28 hours, and at 48 hours after injection of alloxan. The populations of 5 were sacrificed at 16, 28 and 48 hours post-injection. Blood sugar levels, adrenal weight, body weight, and adrenal ascorbic acid levels were recorded for all animals. At 28 and 48 hours post-injection, disruption of DAR and concommitant adrenal hypertrophy were significant ($P<.05$), in alloxinated rats over unstressed control rats. Control rats in the populations of 15 were apparently stressed, as indicated by disruption of DAR in the control rats. Adrenal weights of unstressed control rats were 30 mg% of total body weight during the dark phase and 27 mg% during the light phase. Results indicate that advanced diabetes disrupts this rhythm in weight.

A C K N O W L E D G E M E N T S

I wish to acknowledge the guidance and inspiration that Dr. Eugene Krehbiel has given me in the development of this paper. My respect and gratitude also goes to Dr. L. Stephen Whitley for his laboratory assistance. Finally, I wish to acknowledge the laboratory assistance and perserverance of my wife, Jayne.

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LITERATURE REVIEW

The relationship between adrenocortical hormone synthesis and ascorbic acid metabolism has been an object of study since Szent-Gyorgyi (1928) discovered unusually high concentrations of ascorbic acid to be present in the adrenal glands. Further studies proved that the concentration of ascorbic acid is much greater in the fasciculata-reticularis region of the cortex than in the glomerulosa. Since this region of the cortex is concerned primarily with corticoid production, ascorbic acid would appear to have a role in the synthesis of corticoids. Pirani (1952) stated that ascorbic acid has a function in biological oxidation and cellular respiration of tissues because of the ease with which it is oxidized and reduced. Bourne (1934) demonstrated that ascorbic acid in the adrenal lies in close proximity to the golgi apparatus indicating participation in steroid synthesis.

Giroud et al. (1940) and Ratsimamanga (1944) reported that hormone synthesis is dependent upon the presence of ascorbic acid in the adrenals. Kayahan (1951) found that high doses of ascorbic acid resulted in increased production of glucocorticoids. DeNicola et al. (1968) reported that the regenerating adrenal gland produces corticosteroids at a higher rate than does the normal gland per weight of unit tissue and the regenerating adrenal gland has a lower ascorbic acid content. Baca and Chiodi (1965) found that ascorbic acid content is lower in female rats as compared to males. Kitay (1963) demonstrated that corticoid production is higher in females as compared to males. Banerjee et al. (1952) working with scorbutic guinea pigs reported that their results suggested that scorbutic guinea pigs suffer from hypofunction of the adrenal cortex. However,

Prunty (1955) found that corticoid production in scorbutic guinea pigs was not reduced. Pirani (1952) in a review of ascorbic acid and adrenocortical function stated that in ascorbic acid deficient guinea pigs, adrenal cholesterol depletion becomes apparent about the time that the deficient state begins to act as a stress and therefore induces adrenocortical stimulation. He further states that it would appear that the presence of ascorbic acid in high concentrations is not essential for the synthesis and release of cortical hormones.

Cortical hormone synthesis and release is controlled by a variety of factors. Hume and Wittenstein (1950), and DeGroot and Harris (1950) independently reported evidence that the hypothalamus is involved in the regulation of adrenocorticotropic hormone (ACTH) secretion. Since that time several corticotropin releasing factors (CRF) have been isolated and shown to be the actual causative agents for ACTH release from the anterior pituitary. At the present, it may be assumed that a variety of stimuli and/or chemical agents cause release of CRF which results in ACTH secretion from the anterior pituitary.

Sayers et al. (1946) showed that ACTH either endogenous or exogenous will cause depletion of adrenal ascorbic acid and increased corticoid production in vivo. Tepperman (1950), and Ferstl et al. (1951) independently showed that addition of ACTH in vitro to slices of adrenal cortex significantly increased oxygen consumption and depressed the ascorbic acid content. It was also shown that in the hypophysectomized animal, application of stress is not followed by depletion of ascorbic acid and cholesterol from the adrenal cortex (Long, 1947). Monsonyi and Tyslowitz (1943) reported that hypophysectomy is followed by gradual atrophy of the adrenal gland

with gradual depletion of ascorbic acid. Sayers et al. (1944) reported that the concentration of cholesterol remains essentially unchanged in the adrenal of the hypophysectomized animal. These facts indicate that the depletion of adrenal ascorbic acid and of cholesterol is directly associated with increased secretory activity of the adrenal cortex induced by pituitary adrenocorticotropin. Similarly, a normally functioning pituitary is necessary for the maintenance of normal levels of adrenal ascorbic acid.

Although some animals such as the rat can synthesize ascorbic acid, there is no evidence that synthesis of ascorbic acid occurs in the adrenal. Therefore, ascorbic acid in the adrenal must be obtained from the blood stream. Salomon (1942) stated that the adrenal gland must possess a mechanism for the transport and concentration of ascorbic acid. Sharma et al. (1962) found that the transport of ascorbic acid into some isolated tissues such as guinea pig adrenal cortex slices is energy dependent and that the presence of ACTH inhibits the active transport of ascorbic acid. They further concluded that the depletion of adrenal ascorbic acid by ACTH is due to an inhibition of the active uptake of adrenal ascorbic acid produced by the adrenal cortical hormones produced in response to ACTH. This view is supported by DeNicola et al. (1968) who found that the addition of ACTH to an incubation medium containing slices of rat adrenal caused a decrease in the uptake of C^{14} ascorbic acid. They also found that the inhibitory effects of ACTH disappear when puromycin is added to the medium blocking the increase of corticoid production. They concluded that a factor, or factors produced by the adrenal gland in response to ACTH caused inhibition of ascorbic acid uptake and this inhibition may be responsible for the net loss or depletion of adrenal ascorbic acid in vivo after the administration of ACTH.

The Haynes-Berthet theory of ACTH action (1959) stimulated much research in this area. They suggested that ACTH acts on adenyl cyclase to make 3′5′ AMP available for the activation of phosphorylase. The activated enzyme would facilitate glycogenolysis and with the aid of other enzymes make glucose-6-phosphate available ultimately resulting in increased steroidogenesis. DeNicola et al. (1968) reported that 3′5′AMP inhibits ascorbic acid transport. Earp, Watson, and Ney (1970) confirmed these findings and found that cyclohexamide added to a medium containing slices of adrenal gland blocked elevation of cyclic AMP in the adrenal following addition of ACTH and resulted in a decreased inhibition of ascorbic acid transport. These facts indicate that there is a regulatory mechanism at the adrenal level to control corticoid synthesis.

A new aspect of the monitoring of corticoid and ascorbic acid levels is the diurnal variation in cortcoid production. Critchlow et al. (1963) reported that it appears that the rhythm of environmental illumination exerts a phasing influence on the pituitary-adrenal axis which is similar to that described for the pituitary-gonadal system. The shift in the 24-hour pattern of corticoids that followed a change in phase of environmental lighting indicated that the nervous system, probably via the hypothalamo-pituitary axis is involved in mechanisms underlying the circadian rhythm of adrenal cortical function. Furthermore, suppression of the diurnal elevation in corticosteroid levels in male and females following the administration of pentobarbital is compatible with the suggested participation of the nervous system in processes leading to the marked daily excursion in adrenal cortical secretion. Critchlow et al. (1963) also noted sex differences in the diurnal rhythm of resting levels of plasma and adrenal corticosterone,

pituitary ACTH, and circulating leukocytes in rats. They stated that not only is the presence of mature ovaries an important factor in establishing the sex difference, but cyclic variation in ovarian activity leads to marked differences in the corticosteroid production.

Rinne and Kytomaki (1961) noted a diurnal rhythm in the adrenal ascorbic acid values of the rat which displayed an inverse relationship to circulating levels of plasma corticosteroids. Halberg et al. (1959) reported that the corticosterone rhythm leads the rhythm in gross motor activity and lags behind the eosinophil rhythm. They report that an adrenal cycle is preparatory for daily activity and that its period does not depend solely upon environmental control. Mason (1959) working with humans and monkeys felt that the rhythm of corticoid production was so integrated into the general body mechanism that it was not destroyed by a stress, but merely altered or possibly depressed. It is interesting to note that due to its cyclic variation in activity, an alteration in adrenal function would not only signal the onset of a stress, but would also relate that stress to a time of day.

Although the relationship between a stress and the diurnal variation in ascorbic acid levels within the adrenals has not been thoroughly investigated, the phenomenon of its depletion under a variety of stimuli has long served as a parameter of corticoid production. This method, however, can only be applied when an acute stimulation of the adrenals is involved. Van der Vries (1969) reported that slow changes in the functional state of the adrenals are known to occur sometimes without any change in the ascorbic acid content. Also, in rats the resynthesis of adrenal ascorbic acid proceeds at a high rate which accounts for the observation

by Long (1952) that it is not easy to reduce its content by much more than 50%. Pinchot et al. (1949) reported that measurement of adrenal ascorbic acid levels following acute stress constituted a better index of adrenal function than measurement of adrenal ascorbic acid levels during a prolonged stress.

The existence of a functional relationship between the anterior pituitary, the pancreas and the adrenal cortex with respect to the control of carbohydrate metabolism is well established. The role of adrenal cortical hormones in the regulation of carbohydrate metabolism was pointed out by Britton and Silvette (1932). Cannon (1914) was the first to develop the idea that organisms react to unfavorable situations in terms of highly integrated metabolic activities. Selye (1936) reported that an organism responding to a stress followed a predictable sequence of events which he termed as; 1) alarm, in which the body goes into a state of shock with falling temperature, irregular blood sugar levels and depression of nervous function, 2) defense, during which the organism tends to reverse the changes which occurred during the initial alarm reaction, develops an increasing resistance to the stressor and adapts itself to a new situation and 3) exhaustion, when the adaptation acquired during the preceeding stage is lost for one reason or another. Thus the individual roles of the pancreas, pituitary and adrenal glands in the control of glucose metabolism, the response of ciuculating glucose levels to stress and the interaction of these three glands in stress conditions seems evident.

Diabetes, with hyperglycemia and its related disturbances of fluid electrolytes, carbohydrates, protein and fat metabolism would seem to be a considerable stress on an organism. It is unique in the sense that it

would disrupt the general adaptive syndrome itself with its imbalance of blood sugar levels. One of the main functions of the glucocorticoids released under stimulus of ACTH is to increase the glucose level in the blood. In diabetes therefore, glucocorticoids would only augment the blood sugar imbalance.

Long and Lukens (1936) demonstrated the improvement of diabetes which results from adrenalectomy and Ingle (1944) established the diabetogneic potency of pure cortical hormones. Although they elevate the diabetic state there is evidence to prove increased production of corticoids in diabetes. Bennet and Koneff (1946), and Applegarth (1949) reported enlargement and increased function of the adrenal cortex of rats made diabetic with alloxan. Kalant (1955) found that alloxan diabetic rats excreted considerably more of both biologically active and inactive corticoids than did normals. Fasting had no effect on the excretion of intact animals, but lowered that of the diabetics to almost normal levels. Field (1955) prevented adrenal cortical hypertrophy in diabetic rats by regulating the diabetes with insulin, only the adrenals of the untreated diabetic rats showed depletion of lipids and cholesterol indicating marked cortical hypertrophy. Saba and Hoet (1962) produced hypophyseal inhibition with fluorocortisol and found that the increase in peripheral corticosterone levels following alloxan injection was prevented as well as post alloxanic hyperglycemia. They interpreted this as an indication that alloxan produces an adrenal stress reaction. Devercerski and Frawley (1963) stated that it appears that the increase in adrenal steroid production observed in rats with acutely induced diabetes results from a pituitary-adrenal stress mechanism, while that occuring in the chronic diabetic state is due primarily to the

persistance of a generally altered metabolic state and all of its associated dysfunctions. In the animal with chronic diabetes, metabolic adaptation develops gradually and causes little change in adrenal cortical function.

It would seem therefore, that ascorbic acid levels in the adrenals of alloxan diabetic rats would reflect increased corticoid production. Rose (1951) found a marked increase in adrenocortical activity in uncontrolled alloxan diabetic rats with subsequent fall in adrenal ascorbic acid levels. Dury (1952) found 65 hours after administration of alloxan an increase in adrenal weight and ascorbic acid content of the adrenal gland in rats. However, glucose administration to both control and alloxan diabetic animals resulted in a rapid fall in ascorbic acid content. It is possible that after 65 hours the ascorbic acid level of the adrenal was elevated due to an increased synthesis of the vitamin and the administration of glucose signaled another more severe stress to the animals. Shepard et al. (1952) reported normal ascorbic acid levels in the adrenal glands of alloxan diabetic rats. These investigators did not monitor ascorbic acid levels in the adrenal until 7 days after alloxan administration however. Rose (1951) postulated that adrenal ascorbic acid values are returning to normal levels by 96 hours post-injection of alloxan following a drop during the first 48 hours.

No studies have been directed at following the ascorbic acid level within the adrenal during the period required to produce diabetes from the time of alloxan injection. Also, there is no information as to the effect of alloxan diabetes on the diurnal rhythm of adrenal ascorbic acid. The purpose of this study was to follow adrenal ascorbic acid levels, blood sugar levels and adrenal weights in a population of normal and

alloxinated animals housed separately and together. This provided the opportunity to observe the effects of housing severely diabetic animals with normal controls.

MATERIALS AND METHODS

Two hundred female albino rats were used in this study. The rats were divided into two experimental groups. Group I included one hundred and fifty rats divided into populations of fifteen. Each population of fifteen was subdivided into groups of 7 or 8 animals and housed in two separate cages with one or two control rats per cage respectively. All animals were marked by ear clipping. Group II included fifty rats divided into populations of 5 per cage. Each cage of 5 animals was composed of all control or all alloxinated animals. All cages used contained 13477 cm^3 of space. The rats were housed in the animal room at the Zoology Department of Eastern Illinois University. Temperature in the room was maintained at approximately 75°F. A twelve hour light day, from 7 AM to 7 PM was maintained throughout the experiment by an automatic timer. Rats were fed a standard diet, crude protein (min) 24.0%, crude fat (min) 4.0%, and crude fiber (max) 4.5%. Rats were fed and watered daily.

Injection of experimentals in Group I took place at 8 AM after an overnight fast. Experimental rats were weighed, etherized and given a subcutaneous dose of 175 mg/kg of alloxan monohydrate in a 5% concentration of citrate-phosphate buffer at pH 4. The alloxan was manufactured by Mann Research Laboratories. Control rats were weighed, etherized, and placed back into cages with the alloxinated animals. All rats were fed post-injection, or in the case of the controls, post-etherization. In Group II, the controls and experimentals were placed in separate cages from the time they arrived. None of the rats were marked by ear clipping, all rats were fed normally prior to the time of injection and only the

experimentals were etherized just prior to their injection. Alloxinated animals in Group II also received a dose of 175 mg/kg.

The rats were not disturbed from the time of injection until sacrifice of the animals took place, other than normal daily feeding activity. Rats in Group I were sacrificed by populations of fifteen every four hours from the time of injection for twenty-eight hours and a population was sacrificed at forty-eight hours post-injection. Repeat studies were run on the twenty-four and forty-eight hour post-injection readings in Group I. Rats in Group II were sacrificed by cages of five controls or alloxinated animals at sixteen, twenty-eight, and forty-eight hours post-injection. All animals were weighed prior to sacrifice, then rapidly decapitated by guillotine.

After the blood was obtained for blood sugar determinations, the adrenals were removed, all fat was removed from the adrenal, and the adrenals were weighed to the nearest .01 mg. The adrenals were then homogenized in a 6% trichloroacetic acid in a glass grinding tube. Ascorbic acid determinations were done according to the method of Roe and Keuther (1943). Readings were taken on a Bausch and Lomb spectrophotometer at 515 nm in a 1 cm cuvet. Values were obtained according to the formula;

$$\text{mg ascorbic acid/100 mg adrenal} = \frac{\text{OD} \times \text{Factor} \times \text{Dilution} \times 100}{\text{adrenal weight}}$$

Blood sugar determinations, after deproteinization by the method in Davidson and Wells (1953), were made using a Glucose Stat Pack manufactured by Cal Biochem. Readings were taken on a Bausch and Lomb spectrophotometer at 314 nm in a 1cm cuvet. Values were obtained according to the formula; glucose in mg% = DA after 5 min x 435 x dilution factor, DA being the observed change in absorbance after the reaction has proceeded to completion.

This method accurately determines up to 400 mg% glucose in the blood. Above this level modifications in the procedure require further reagents. Since 400 mg% is obviously in the diabetic range, no determinations were made above this level. The Students T Test was run on blood sugar levels, adrenal weights and adrenal ascorbic acid levels to determine the significance of results.

O B S E R V A T I O N S

Study Group I

In this study one hundred and fifty rats were divided into populations of fifteen. Each population was comprised of twelve alloxinated and three control rats. A population was sacrificed every four hours from the time of injection until twenty-eight hours post-injection. A population was also sacrificed at forty-eight hours post-injection. Repeat studies were run for the twenty-four and forty-eight hour post-injection readings.

Blood Sugar

Blood sugar values in the alloxinated animals averaged 338 mg% at four hours (Table I) and never went below diabetic levels at any of the periods values were taken in the forty-eight hours. At forty-eight hours the blood glucose average for alloxinated animals was 390 mg% (Table VIII). The glucose stat pack used to determine blood glucose levels measured glucose levels up to 400 mg% accurately. Above this level modifications which would have required additional reagents would need to be employed. Therefore, although it was determined that an animal was diabetic, the extent of that diabetes was not determined above 400 mg%. It may be assumed that at forty-eight hours, when the value for all alloxinated animals was 390 mg%, some of the animals had much higher blood glucose levels than that. Control averages for blood glucose varied with the activity of the animal. During the daytime or resting phase controls averaged 92 mg% while during the night or activity phase they averaged 102 mg%. This could be due to increased food intake and increased levels of corticoids during the activity phase. There was a significant difference ($P<.05$) between control and alloxinated animals in blood glucose levels at all times tested.

Adrenal Ascorbic Acid

The mean adrenal ascorbic acid value for control animals in the first twenty-four hours was 469 mg% during the resting phase (8 AM to 8 PM), and 354 mg% during the dark or activity phase (8 PM to 8 AM) (Fig. 1). However, at 12 noon, twenty-eight hours post-injection, the control average is 320 mg% which is 33% lower than at the same time the previous day (Tables I,VII). At forty-eight hours, the control average is 240 mg% which is 36% lower than the value reached at the same time the previous day (Tables VI,VIII).

The average adrenal ascorbic acid value for alloxinated animals was 470 mg% during the resting phase of the first day (Fig. 1). During the dark phase of the first day (8 PM to 8 AM), alloxinated animals averaged 415 mg%. This is higher than the control average for the same time period and is due to failure of alloxinated animals to show depletion of adrenal ascorbic acid with onset of the dark period (Table III). However, at 12 midnight, and 4 PM (Tables IV,V) ascorbic acid values of alloxinated animals were not significantly different than controls. During the resting phase of the second day alloxinated animals averaged 342 mg%, and at forty-eight hours alloxinated animals averaged only 288 mg% (Table VIII). Thus adrenal ascorbic acid values were lower the second twenty-four hours than values reached the previous day. It is of note that a mortality of 50% of the alloxinated animals occurred between twenty-eight and forty-eight hours. It is also interesting that control ascorbic acid values fell during this period of mortality of alloxinated animals.

Adrenal Weight

Adrenals of control animals averaged 27 mg% during the light period of the first day (Fig. 2). During the dark period (8 PM to 8 AM) adrenal weight rose to 32 mg%. During the light period of the second day control animals had average adrenal weights of 30 mg%. Thus adrenals of controls were heavier than alloxinated animals in the second twenty-four hour period. At twenty-eight hours adrenal weights of control animals were 16% heavier than at the same clock time the previous day (Tables I,VII). At 8 AM forty-eight hours post-injection, adrenal weights of controls were 10% higher than at the same clock time the previous day.

Results from this study indicate that adrenal hypertrophy is evident four hours after injection in alloxinated animals with adrenals averaging 31 mg% as opposed to 27 mg% in controls (Table I). Generally adrenals of alloxinated animals were slightly heavier than controls until 12 hours after injection when adrenals of alloxinated animals were lower in weight than controls though not significantly so. At sixteen hours (Table IV) alloxinated animals again averaged higher adrenal weights, but were again at control levels at twenty hours (Table V). From twenty-four hours through forty-eight hours however, alloxinated animals displayed higher adrenal weights than control animals. Hypertrophy is most evident at forty-eight hours when alloxinated animals averaged 34.8 mg% as opposed to 30.1 mg% in controls (Table VIII).

Study Group I

Table I

Time: 12:00 noon 4 hours post-injection

	Control $N_1 = 3$		Alloxinated $N_2 = 12$		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	186.0	7.93	175.1	5.0	
Ad wt (mg)	51.0	5.68	55.5	7.3	
Ad wt/Body wt (mg%)	27.3	2.3	31.1	4.7	1.32
Bl sugar (mg%)	93.0	35.0	338.8	120.6	3.41*
Asc acid (mg%)	474.5	113.8	443.3	82.9	0.544*

Table II

Time: 4:00 PM 8 hours post-injection

	Control $N_1 = 3$		Alloxinated $N_2 = 12$		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	182.6	3.2	182.6	10.0	
Ad wt (mg)	51.0	5.0	55.9	6.9	
Ad wt/Body wt (mg%)	27.6	3.0	30.0	3.5	1.34
Bl sugar (mg%)	85.0	31.0	263.6	140.3	2.19 *
Asc acid (mg%)	555.0	104.7	497.6	103.1	0.87 *

* Denotes significance at 95% level

Table III

Time: 8:00 PM 12 hours post-injection

	Control N_1 = 3		Alloxinated N_2 = 12		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	199.6	4.1	188.9	12.2	
Ad wt (mg)	65.0	5.5	53.8	6.8	
Ad wt/Body wt (mg%)	31.6	2.5	28.0	4.1	1.40
Bl sugar (mg%)	94.0	7.2	315.8	92.3	4.04 *
Asc acid (mg%)	351.7	72.0	524.7	105.2	2.66 *

Table IV

Time: 12:00 midnight 16 hours post-injection

	Control N_1 = 3		Alloxinated N_2 = 10		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	173.0	17.0	171.4	5.3	
Ad wt (mg)	55.0	3.6	58.2	6.8	
Ad wt/Body wt (mg%)	31.6	2.0	33.5	4.0	0.65
Bl sugar (mg%)	107.6	24.0	269.4	120.2	2.26 *
Asc acid (mg%)	317.9	40.2	322.9	53.1	0.15

* Denotes significance at 95% level

Table V

Time: 4:00 AM 20 hours post-injection

	Control N_1 = 3		Alloxinated N_2 = 12		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	165.6	3.7	172.2	8.7	
Ad wt (mg)	57.3	8.3	59.6	8.2	
Ad wt/Body wt (mg%)	34.3	5.0	34.1	4.3	0.06
Bl sugar (mg%)	105.3	22.4	377.5	46.5	9.7 *
Asc acid (mg%)	404.6	138.7	400.1	76.6	0.05

Table VI

Time: 8:00 AM 24 hours post-injection

	Control N_1 = 8		Alloxinated N_2 = 22		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	181.0	14.9	182.1	9.5	
Ad wt (mg)	50.1	6.9	59.6	7.9	
Ad wt/Body wt (mg%)	27.3	3.0	32.3	4.7	2.79 *
Bl sugar (mg%)	100.7	24.8	336.3	104.7	6.23 *
Asc acid (mg%)	370.6	93.5	349.4	72.2	0.67

* Denotes significance at 95% level

Table VII

Time: 12:00 noon 28 hours post-injection

	Control N_1 = 3		Alloxinated N_2 = 8		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	182.6	33.8	212.1	5.0	
Ad wt (mg)	61.0	10.1	72.1	7.0	
Ad wt/Body wt (mg%)	32.6	1.5	33.5	3.5	0.21
Bl sugar (mg%)	79.6	12.7	310.1	132.8	2.94 *
Asc acid (mg%)	320.8	86.1	288.7	41.3	0.98

Table VIII

Time: 8:00 AM 48 hours post-injection

	Control N_1 = 8		Alloxinated N_2 = 18		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	190.0	11.7	168.3	11.4	
Ad wt (mg)	57.6	7.8	59.5	9.5	
Ad wt/Body wt (mg%)	30.1	4.7	34.8	4.6	2.4 *
Bl sugar (mg%)	109.3	21.0	390.8	29.1	24.5 *
Asc acid (mg%)	240.8	44.2	288.9	107.7	1.2

* Denotes significance at 95% level

Study Group I

Fig. 1. Mean adrenal mg% ascorbic acid levels

Control vs. Alloxinated animals

STUDY GROUP I

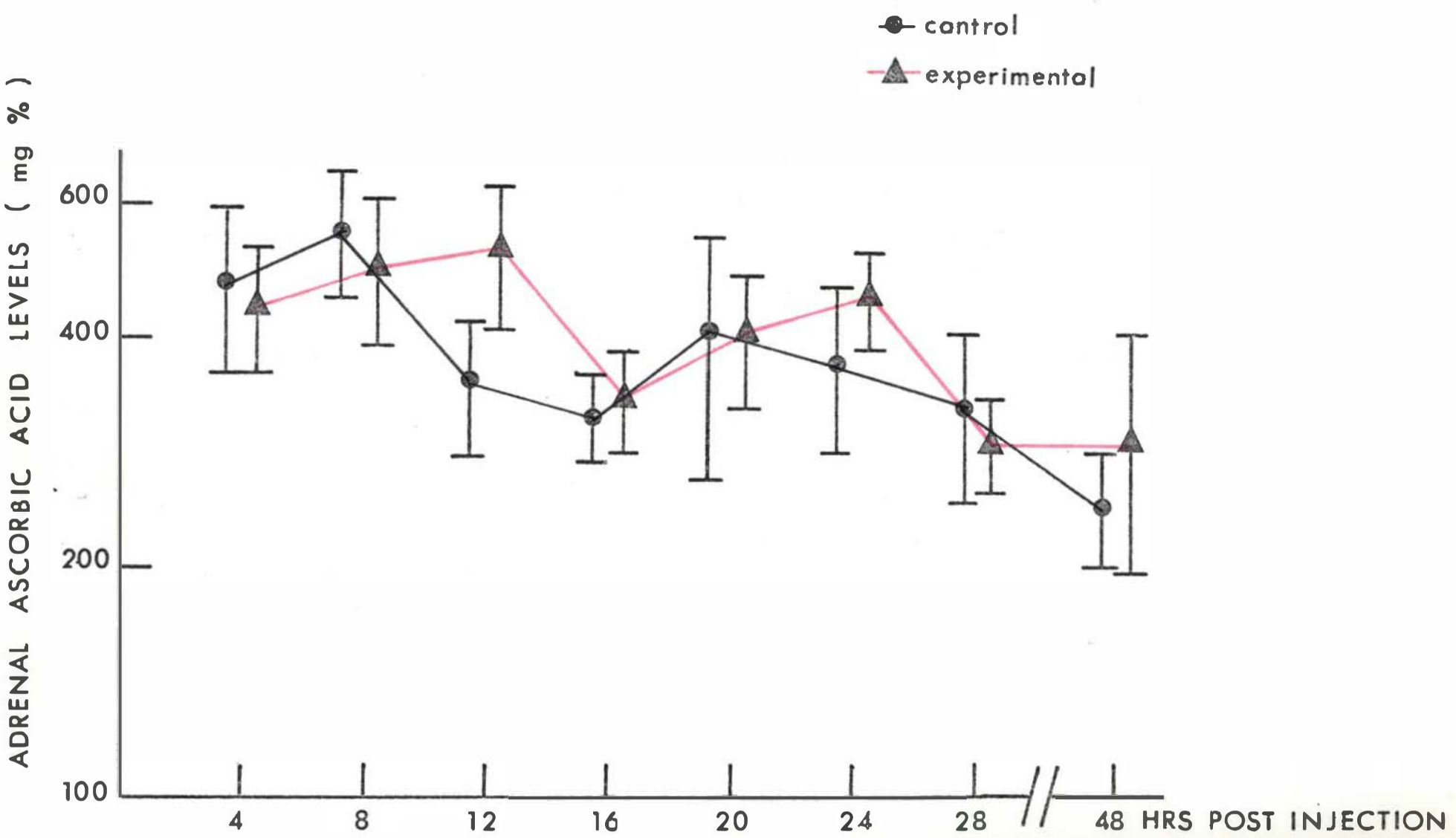

FIGURE 1

Study Group I

Fig. 2. Mean adrenal weight expressed as mg% body weight

Control vs. Alloxinated animals

STUDY GROUP I

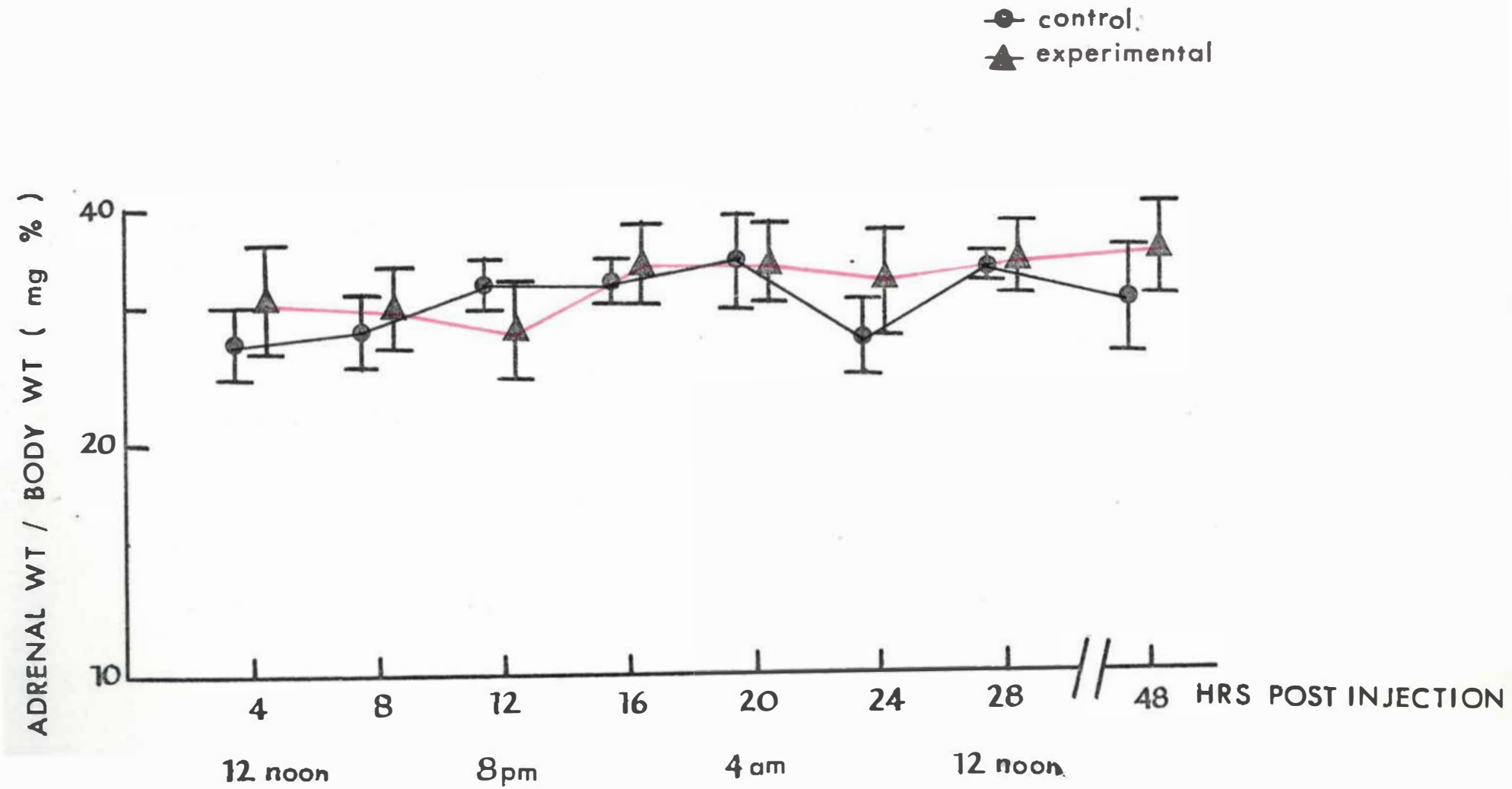

FIGURE 2

Study Group II

In this study alloxinated animals were housed separately from controls with five animals per cage. Animals were sacrificed at sixteen, twenty-eight and forty-eight hours post-injection.

Blood Sugar Levels

Blood sugar levels for controls were 98 mg% at midnight, 92 mg% at 8 AM, and 95 mg% at 12 noon (Tables IX,X,XI). There was not a variation in glucose levels according to the activity of the animal. Values for alloxinated animals were 241 mg% at midnight, 388 mg% at 12 noon, and 354 mg% at 8 AM (Tables IX,X,XI). There were significant differences ($P<.05$) between control and alloxinated blood glucose levels at all times tested.

Adrenal Ascorbic Acid

Control animals averaged 326 mg% as opposed to 243 mg% in alloxinated animals at sixteen hours (Table XI). At twenty-eight hours control animals averaged 465 mg% as opposed to 290 mg% in alloxinated animals (Table X). Finally at forty-eight hours control animals averaged 320 mg% as opposed to 250 mg% in alloxinated animals (Table IX). At all times values were taken, control adrenal ascorbic acid levels were significantly higher ($P<.05$) than alloxinated animals.

Control adrenal ascorbic acid values were similar for both study group I and II at midnight, sixteen hours post-injection (Tables IV,XI). However, at twelve noon, twenty-eight hours post-injection, controls in study group II averaged 465 mg% as opposed to 320 mg% in controls of study group I (Tables VII,X). At forty-eight hours controls in study group II averaged 320 mg% as opposed to 240 mg% in controls of study group I (Tables VIII,IX).

Adrenal Weight

Adrenal weights for control animals were 27 mg% at sixteen hours, 24 mg% at twenty-eight hours, and 29 mg% at forty-eight hours (Tables IX,XI). Adrenal weights for alloxinated animals averaged 33 mg% at sixteen hours, 34 mg% at twenty-eight hours, and 40 mg% forty-eight hours post-injection (Tables VI,X). Adrenal weights of controls were significantly lower than adrenal weights of alloxinated animals each time values were taken (Fig. 4).

Study Group II

Table IX

Time: 8:00 AM 48 hours post-injection

	Control N_1 = 10		Alloxinated N_2 = 5		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	209.0	28.3	179.6	26.4	
Ad wt (mg)	60.0	13.0	73.0	16.4	
Ad wt/Body wt (mg%)	29.0	3.0	40.0	7.3	9.5 *
Bl sugar (mg%)	92.0	22.6	354.0	74.0	18.4 *
Asc acid (mg%)	320.0	80.7	250.0	69.0	14.6 *

Table X

Time: 12:00 noon 28 hours post-injection

	Control N_1 = 10		Alloxinated N_2 = 5		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	205.0	15.8	202.0	4.4	
Ad wt (mg)	50.0	13.4	69.0	9.9	
Ad wt/Body wt (mg%)	24.0	6.3	34.0	4.8	3.1 *
Bl sugar (mg%)	95.0	24.0	388.0	26.8	108.0 *
Asc acid (mg%)	465.3	131.0	290.0	54.5	30.9 *

* Denotes significance at 95% level

Table XI

Time: 12:00 midnight 16 hours post-injection

	Control $N_1 = 5$		Alloxinated $N_2 = 10$		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	221.0	19.6	222.0	18.9	
Ad wt (mg)	60.4	6.6	71.0	7.2	
Ad wt/Body wt (mg%)	27.0	1.2	31.0	4.6	3.8 *
Bl sugar (mg%)	98.8	19.1	241.0	113.0	28.4 *
Asc acid (mg%)	326.0	22.3	243.0	50.6	2.7 *

* Denotes significance at 95% level

Study Group II

Fig. 3. Mean adrenal mg% ascorbic acid levels

Control vs. Alloxinated animals

STUDY GROUP II

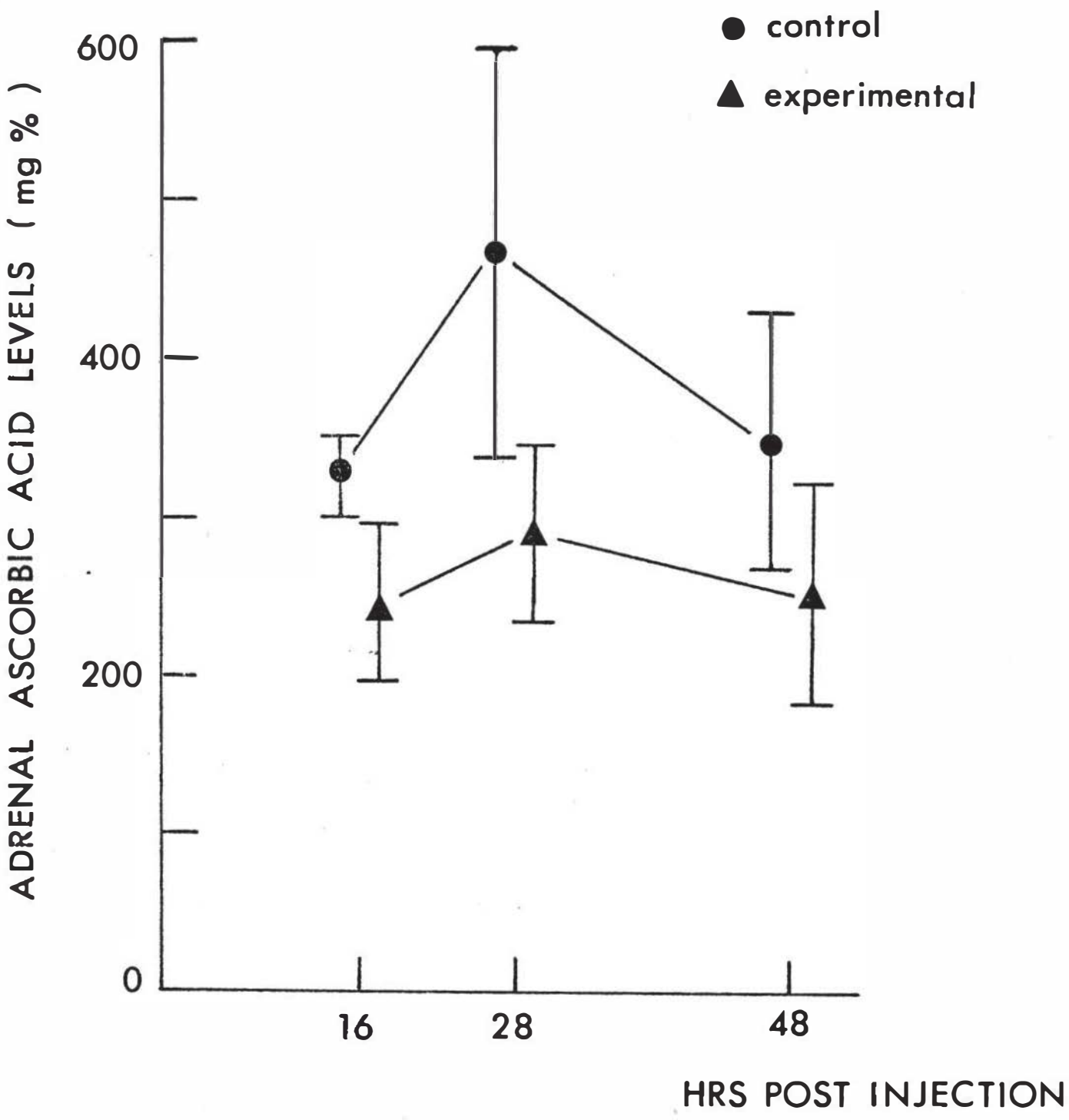

FIGURE 3

Study Group II

Fig. 4. Mean adrenal weight expressed as mg% body weight

Control vs. Alloxinated animals

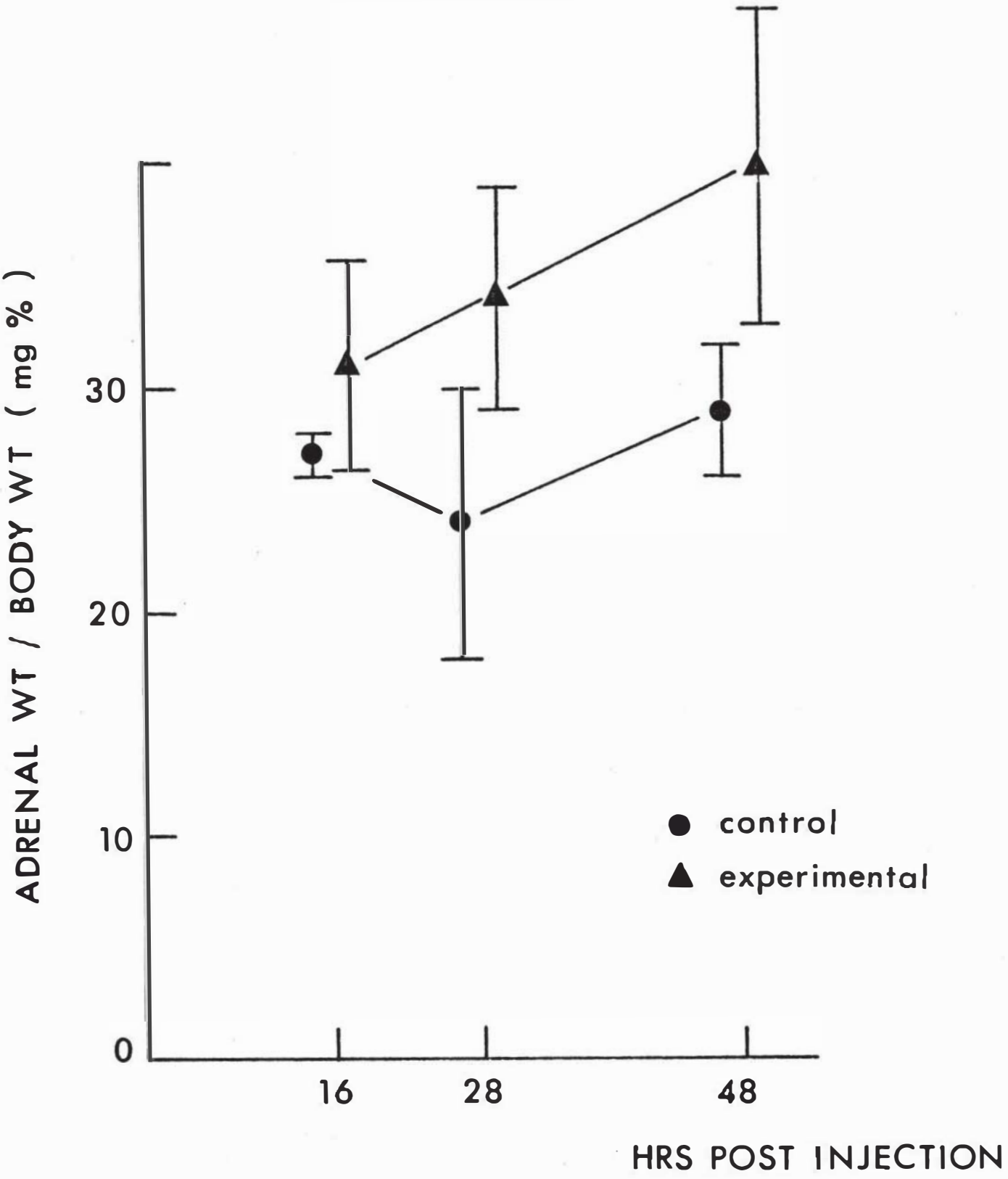
STUDY GROUP II
ADRENAL WT / BODY WT (mg %)
30
20
10
0
16
28
48
HRS POST INJECTION
control
experimental

FIGURE 4

Combined Values

Groups I and II

Table XII

Time: 12:00 noon 4 hours post-injection

	Control N_1 = 16		Alloxinated N_2 = 12		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	208.0	22.0	171.0	6.7	
Ad wt (mg)	55.0	10.8	55.0	7.4	
Ad wt/Body wt (mg%)	26.0	6.2	32.0	4.8	7.3 *
Bl sugar (mg%)	98.0	24.6	338.0	120.6	60.5 *
Asc acid (mg%)	469.0	143.0	420.0	86.1	30.7 *

Table XIII

Time: 12:00 midnight 16 hours post-injection

	Control N_1 = 8		Alloxinated N_2 = 20		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	205.0	30.7	196.0	29.4	
Ad wt (mg)	58.0	6.7	65.0	13.9	
Ad wt/Body wt (mg%)	28.0	3.1	33.0	4.3	5.97 *
Bl sugar (mg%)	102.0	19.8	280.0	110.1	44.4 *
Asc acid (mg%)	323.0	26.7	288.0	61.5	26.6 *

* Denotes significance at 95% level

Table XIV

Time: 8:00 AM 24 hours post-injection

	Control N_1 = 13		Alloxinated N_2 = 22		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	195.0	17.8	175.0	12.1	
Ad wt (mg)	50.0	9.3	60.0	7.9	
Ad wt/Body wt (mg%)	27.0	2.6	34.0	5.0	8.86 *
Bl sugar (mg%)	97.0	24.0	336.0	90.7	83.5 *
Asc acid (mg%)	374.0	88.0	348.0	72.1	8.4 *

Table XV

Time: 12:00 noon 28 hours post-injection

	Control N_1 = 16		Alloxinated N_2 = 13		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	208.0	22.0	207.0	6.9	
Ad wt (mg)	55.0	10.8	71.0	8.0	
Ad wt/Body wt (mg%)	26.0	6.2	34.0	3.9	4.1 *
Bl sugar (mg%)	98.0	24.6	336.0	107.8	77.9 *
Asc acid (mg%)	469.0	143.0	289.0	130.6	44.5 *

* Denotes significance at 95% level

Table XVI

Time: 8:00 AM 48 hours post-injection

	Control N_1 = 18		Alloxinated N_2 = 23		
	$\bar{X}$	SD	$\bar{X}$	SD	T value
Body wt (gr)	195.0	17.8	170.0	14.5	
Ad wt (mg)	50.0	9.3	62.0	13.2	
Ad wt/Body wt (mg%)	27.0	2.6	36.0	5.7	14.0 *
Bl sugar (mg%)	97.0	24.0	382.0	43.2	156.9 *
Asc acid (mg%)	374.0	88.0	280.0	109.0	30.6 *

* Denotes significance at 95% level

Combined Averages Study Groups I & II

Fig. 5. Mean adrenal mg% ascorbic acid levels

Control vs. Alloxinated animals

STUDY GROUPS I AND II

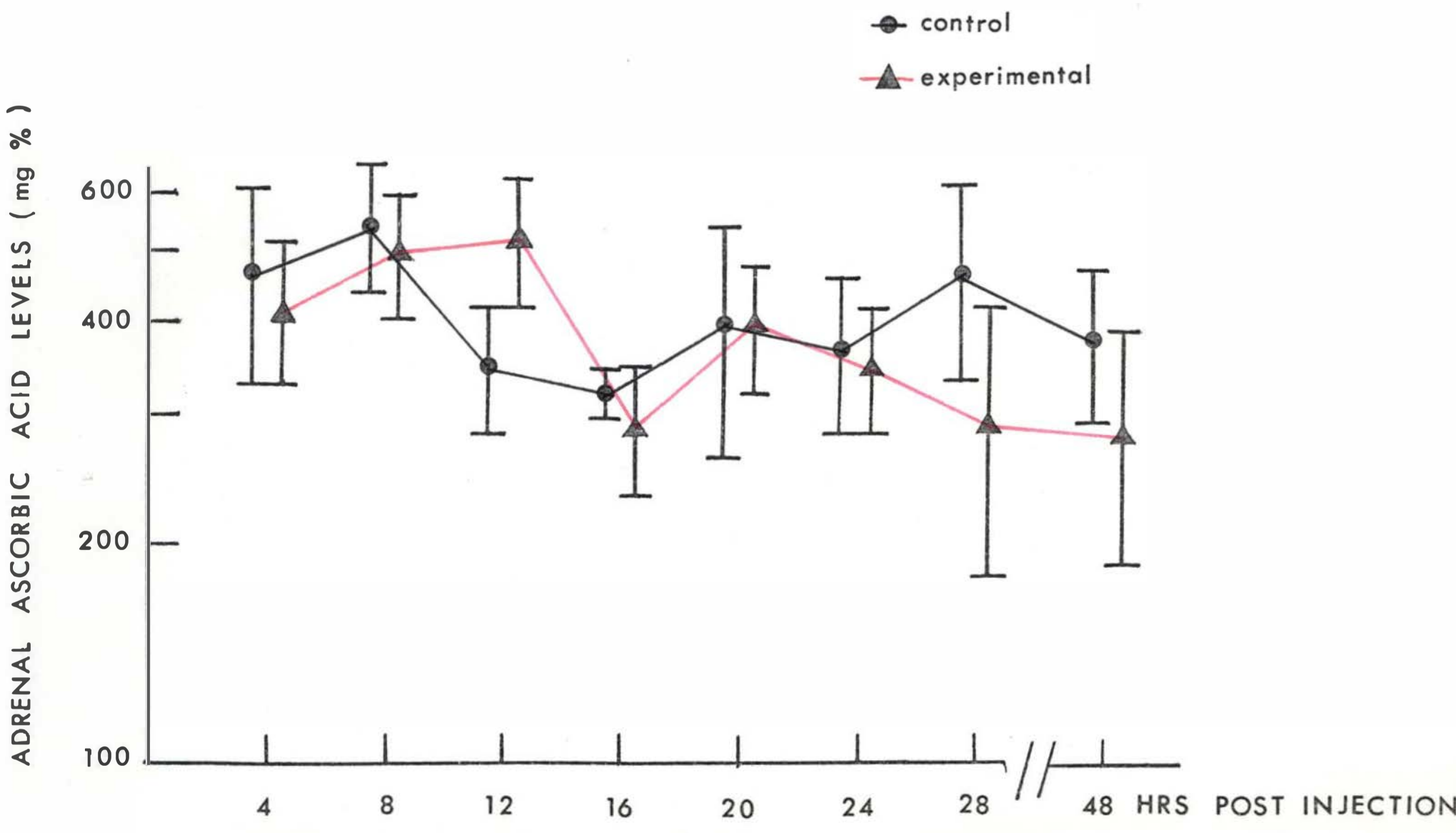

FIGURE 5

Combined Averages Study Groups I & II

Fig. 6. Mean adrenal weight expressed as mg% body weight

Control vs. Alloxinated animals

STUDY GROUPS I AND II

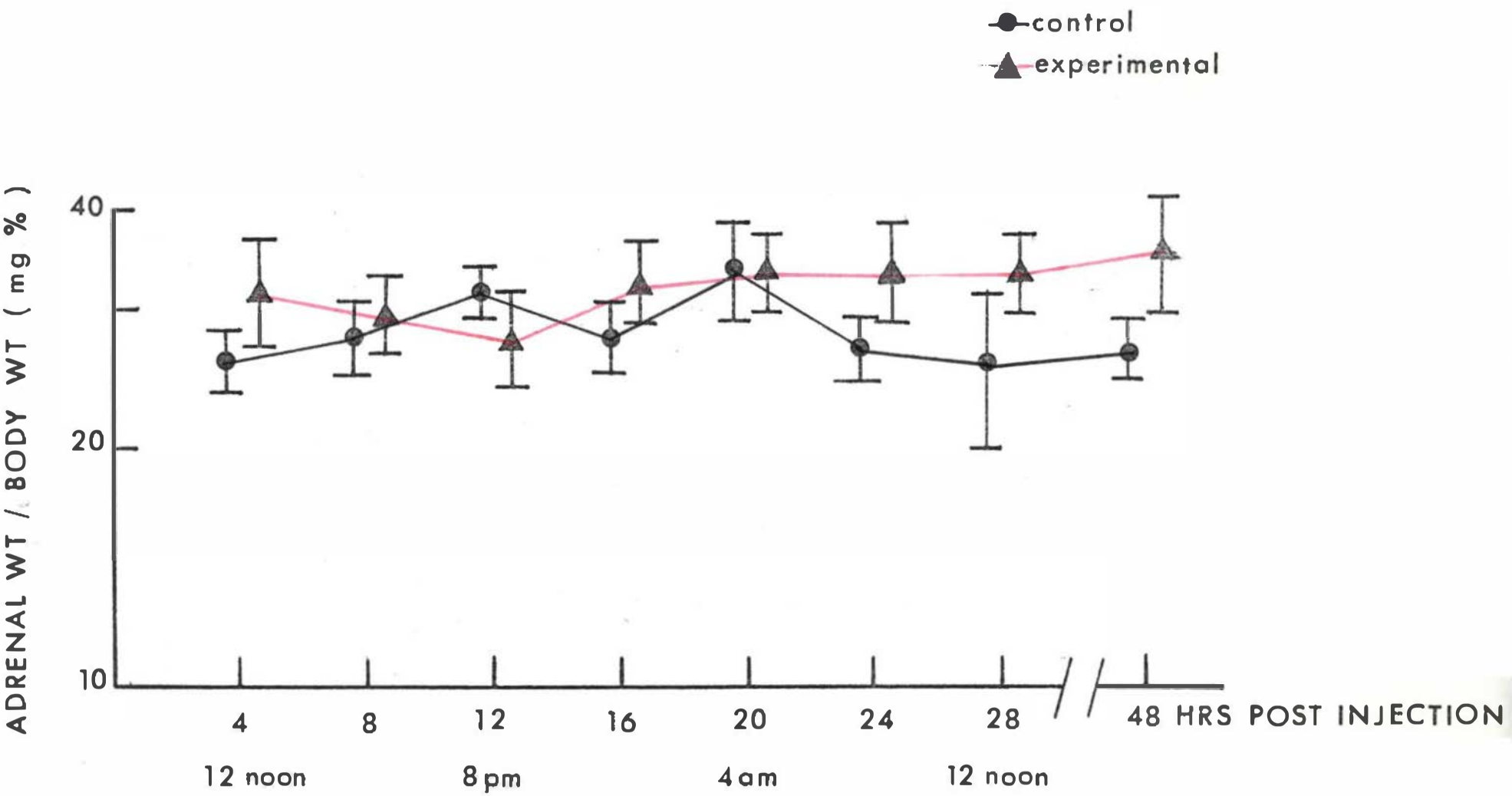

FIGURE 6

Combined Averages Study Groups I & II

Fig. 7. Mean diurnal adrenal mg% ascorbic acid levels

Unstressed controls

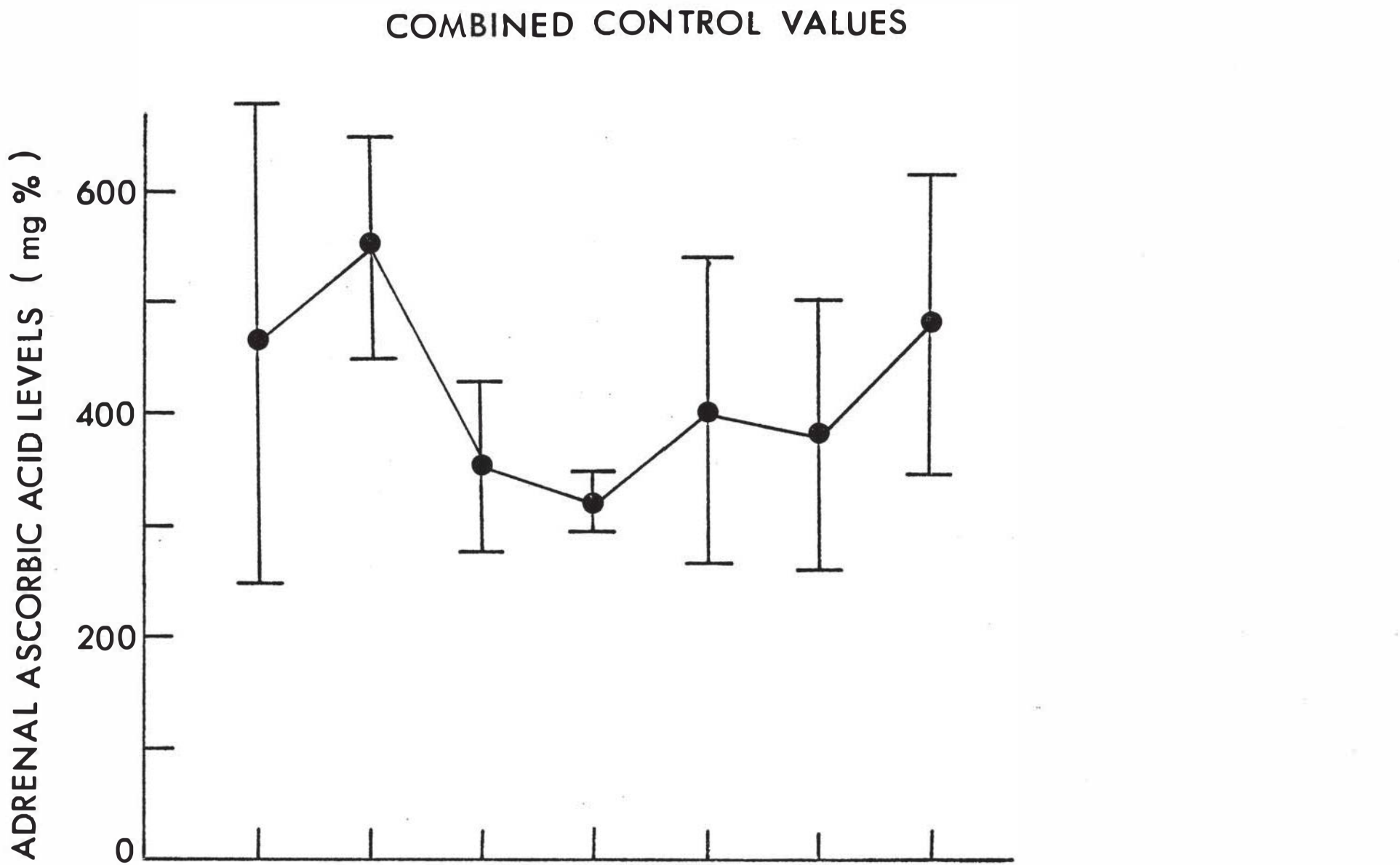
COMBINED CONTROL VALUES
ADRENAL ASCORBIC ACID LEVELS (mg %)
600
400
200
0
4
12 noon
8
12
8pm
16
20
4 am
24
28
12 noon
HRS POST INJECTION

FIGURE 7

Combined Averages Study Groups I & II

Fig. 8. Mean diurnal adrenal weight expressed as mg% body weight

Unstressed Controls

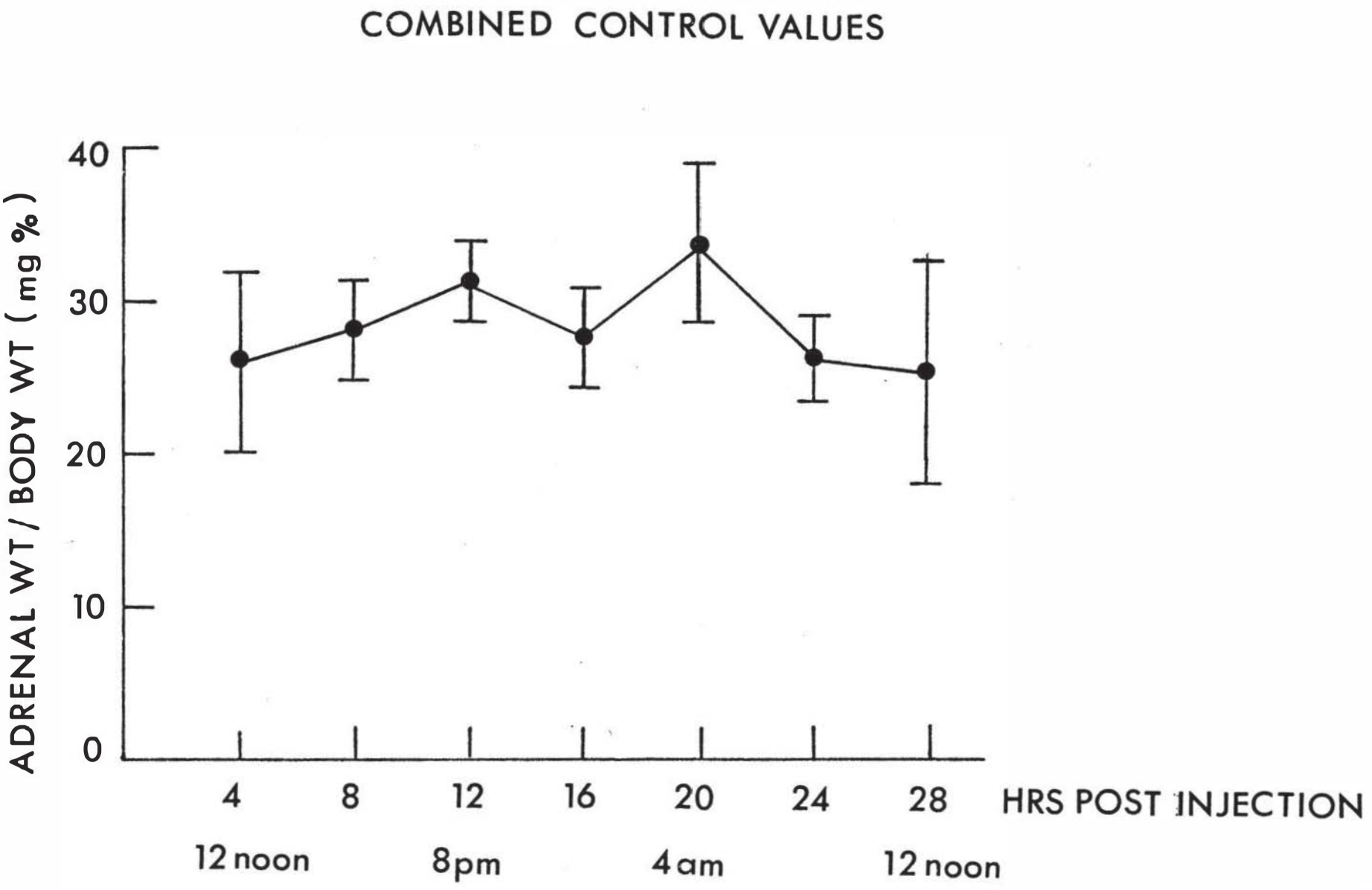
COMBINED CONTROL VALUES
ADRENAL WT / BODY WT (mg %)
40
30
20
10
0
4
8
12
16
20
24
28
HRS POST INJECTION
12 noon
8pm
4 am
12 noon

FIGURE 8

D I S C U S S I O N

In this study, a diurnal rhythm of adrenal ascorbic acid is revealed with an average of 430 mg% from 8 AM to 8 PM corresponding to the light period and 350 mg% from 8 PM to 8 AM corresponding to the dark period (Fig. 7). Although plasma corticosteroids were not recorded in this study, a fall in adrenal ascorbic acid and a rise in adrenal weight is associated with onset of the dark period and increased motor activity. Critchlow et al. (1963) reported that corticoid levels in the blood of rats reached peak levels just prior to onset of the dark period then declined through the dark period to level off at the onset of the light period. Halberg et al. (1959), working with mice, also reported peak levels of serum corticosterone just prior to onset of the dark period indicating that corticosterone rhythm leads in phase the rhythm in gross motor activity. Rinne and Kytomaki (1961) reported a diurnal rhythm of adrenal ascorbic acid in the rat which displayed an inverse relationship to previously reported patterns of serum corticosterone. Therefore, adrenal ascorbic acid levels appear to be an accurate indicator of the physiology of the resting adrenal.

Rinne and Kytomaki (1961) also reported an increase in adrenal weight at midnight and related this increase in weight to increased mitosis in the cortex at this hour. Adrenal weights were higher in resting controls in this study during the dark period, averaging 30 mg% as opposed to the light period with an average of 27 mg% (Fig. 8). Brodish and Long (1960) using unilateral adrenalectomy as a stress noted that the increase in size and weight of the remaining adrenal was due to an increase in the wet weight and not the dry weight of the remaining adrenal.

They stated that the rapid increase in weight was due to fluid accumulation in the remaining adrenal. There is a hypertrophy of the adrenal at estrus not associated with increased mitosis. Hypertrophy *per se* may be restricted to denote greater bulk through size, but not in number of the individual tissue elements. The rhythm of adrenal weight noted in this study could be related to increased fluid content and/or lipid content in the cells of the cortex rather than mitotic activity. The weight would not decline during the light period if it was due to increased numbers of cells. It is also possible that the hypertrophy of the adrenals of diabetic animals noted in this study is related to increased fluid content rather than an actual increase in tissue elements. This certainly deserves further attention.

If the rhythm of adrenal ascorbic acid and adrenal weights in this study are compared one finds that the increased adrenal weight is associated with a decreased ascorbic acid content of the adrenal (Figs. 7,8). Critchlow *et al*. (1963) reported that under conditions of a 0400-1800 lighting schedule peak adrenal cortical activity occurred between 1500-1900 hours. In this study under conditions of a 0700-1900 lighting schedule it would appear that peak cortical activity occurs between 2000-2400 hours. It is possible that the difference in lighting schedule would be the reason for the difference in peak cortical activity between this study and earlier results of Critchlow *et al*. (1963).

The controlling factors of the diurnal rhythm of adrenal function are undoubtedly a complex interaction of environmental influence on endogenous control systems involving the hypothalamus, higher brain centers, gonads, and the adrenal itself. It is not surprising that Mason (1959) reported

that the diurnal rhythm of adrenal activity was so integrated in the entirety of body function that it could not be destroyed by a stress, but merely altered or possibly depressed. However, as evident in this study, the disruption of the rhythm (Figs. 1,5) would seem to be an accurate indicator of increased adrenal function and onset of a severe stress.

Blood Sugar Values

The blood glucose average in controls was 95 mg% for the light period and 100 mg% for the dark period. Hemolysis of many blood samples had the effect of raising the reading on the photometer and the net result was higher blood sugar values than the actual for many animals. Therefore there may be a greater difference between the light and dark periods as regards blood glucose than was found. Blood glucose values for alloxinated animals were above 250 mg% at all periods tested. Results from this study agree with previous findings of a correlation between high blood sugar levels and increased adrenal activity.

Adrenal Activity of Alloxinated Animals

This correlation is evident in both adrenal ascorbic acid levels and adrenal weights of alloxinated animals. Saba and Hoet (1962) using a dose of 40 mg/kg of alloxan on rats found a rapid increase in corticosterone levels within one hour post-injection of alloxan. This increase then returned to normal levels by six hours post-injection, but was again elevated three days post-injection. They reported that after the first adrenocortical stimulation by alloxan a chronic state is eventually perpetuated in which high levels of glucocorticoids occur associated with hyperglycemia.

Results from this study show a decreased ascorbic acid content and higher adrenal weight at four hours post-injection (Figs. 5,6). At twelve hours post-injection however, adrenal ascorbic acid levels were higher in alloxinated animals and adrenal weights were lower in alloxinated animals compared to controls. This could either mark the recovery phase of the initial alarm reaction or the inability of the alloxinated animals to react to onset of the dark period at that time. However, at sixteen and twenty hours, control and alloxinated animals had very similar adrenal ascorbic acid and adrenal weight values. This would seem to indicate that the alloxinated animals had reached some sort of stability even though their blood sugar levels were increasing. At twenty-eight hours, elevated adrenal weight and decreased adrenal ascorbic acid levels signal the onset of the chronic state described by Saba and Hoet (1962). Hypertrophy of adrenals of alloxinated animals is most evident at forty-eight hours when alloxinated animals had an average adrenal weight of 36 mg% as opposed to 27 mg% in controls (Table XVI).

Field (1955) found that insulin treated alloxinated animals did not exhibit adrenal hyperactivity. This also indicated that alloxan had no direct effect on the adrenal cortex. Bennet and Koneff (1946) observed that hypertrophy of the adrenal cortex following establishment of alloxan diabetes persisted for at least a month and could not be due to an acute effect of alloxan. Saba and Hoet (1962) using fluorocortisol to inhibit the hypophyso-adrenal axis suppressed the stress reaction and hyperglycemia due to alloxan. Lukens (1948) in a review of alloxan and alloxan compounds described an initial hypoglycemia due to alloxan injection. Although blood sugar levels four hours post-injection of alloxan in this study

were above 250 mg%, it could have followed an initial hypoglycemia related to the alarm reaction of alloxan injection. Hypoglycemia would result in increased corticoid production. Kalant (1955) stated that fed diabetic rats excreted considerably larger amounts of corticoids than did normals, while fasted diabetic rats returned to normal levels of urine corticoids. He stated that it is possible that glucose excretion caused an osmotic diuresis which in turn washed out corticoids which might have ordinarily been reabsorbed. Field (1955) stated that while lipid depletion did not occur in the fasciculata-reticularis region of the adrenal cortex of insulin treated alloxinated animals, there was a marked depletion of lipid from the glomerulosa of both insulin-treated and untreated diabetic animals indicating that some renal damage had occured. Kalant (1955) stated that renal damage does occur within the first few days after alloxination, but if in the alloxan-treated rats there existed a tubular defect in the resorption of corticoids one would not expect fasting to alter the excretion.

Instead, corticoid excretion was related to urine volume, which increases as glucose appears in the urine. It is well known that polyuria ia a symptom of diabetes and this is probably one of the reasons for the depletion of lipids in the glomerulosa as the animal attempts to counteract dehydration and lowered blood volume. High blood sugar levels result in increased urine volume and excretion of corticoids, thus control of the adrenal output is drastically altered at the adrenal where feedback of corticoids on the adrenal is reduced. Increased need for glucose in peripheral tissue would result in increased release of ACTH also. These factors would in turn result in hyperactivity of the adrenal noted at

twenty-eight and forty-eight hours. If one were to describe the stress reaction due to alloxan in terms of the general adaptive syndrome, it would appear that the alarm reaction occurs within the first four hours. The defense against the diabetic state would seem to occur between eight and twenty-four hours. Exhaustion and death occurred between twenty-eight and thirty-six hours in approximately 50% of the animals in Study Group I and 25% in Study Group II. A dose of 175 mg/kg of alloxan induces a severe stress that may be termed acute both in onset and severity of the diabetes.

Previous studies by Dury (1952), Rose (1951) and Shepard et al. (1952) indicate that ascorbic acid levels return to normal by ninty-six hours. Bennet and Koneff (1946) reported that adrenal hypertrophy persists for a much longer period however. Devercerski and Frawley (1963) noted that in chronic diabetes metabolic adaptation develops gradually and causes little change in adrenal cortical function. They stated that hyperglycemia produced by glucose administered peritoneally caused marked changes in body fluid distribution, while hyperglycemia introduced orally did not. Beyond ninty-six hours the stress of diabetes could be termed chronic. Pinchot et al. (1952) reported that adrenal ascorbic acid levels are not an accurate indicator of adrenal physiology in cases of prolonged stress. Corticoid production and depletion of adrenal ascorbic acid are apparently not correlated under conditions of chronic diabetes. However, adrenal hypertrophy is a consistent phenomenon of the diabetic state.

Results from Study Groups I and II provide evidence that housing control animals with severly diabetic animals has an adverse effect on the control animals. This stress is probably not due to increased population

interaction as alloxinated animals were much less active than control animals. It might be due to something in the urine of alloxinated animals or to a disruption of population interaction. Christian (1964) in his essay on endocrines and behavior describes the relation between adrenal cortical activity and population levels. He stated that the basic stimulus to the endocrine changes are sociopsychological or emotional and not physical in nature. The results obtained in this study support this hypothesis. When control animals are separated from alloxinated animals the stress reaction is abolished in control animals (Figs. 3,4), while alloxinated animals display adrenal hypertrophy and depletion of adrenal ascorbic acid noted in Study Group I.

Combining values of Study Groups I and II (Figs. 7,8), had the net result of not changing the high blood sugar levels, higher adrenal weights and lower adrenal ascorbic acid levels in alloxinated animals. However, combining values for control animals has the result of raising the adrenal ascorbic acid levels twenty-eight hours post-injection of alloxinated animals from 320 mg% (Table VII) to 469 mg% (Table XV). Likewise, at 8 AM forty-eight hours post-injection, combining values raised the ascorbic acid values from 240 mg% in Group I controls (Table VIII) to 374 mg% (Table XVI). Adrenal weights for the same times were also lowered. It would appear that environmental influence including population interaction, visual perception and possibly nasal stimulation has further merit for study in populations of stressed and unstressed animals.

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